Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity

José Mario de Freitas Balanco*¶, Maria Elisabete Costa Moreira†¶, Adriana Bonomo†‡, Patricia Torres Bozza§, Gustavo Amarante-Mendes*, Claude Pirmez§ and Marcello André Barcinski*†

Programmed cell death by apoptosis of unnecessary or potentially harmful cells is clearly beneficial to multicellular organisms [1]. Proper functioning of such a program demands that the removal of dying cells proceed without an inflammatory reaction [2]. Phosphatidylserine (PS) is one of the ligands displayed by apoptotic cells that participates in their noninflammatory removal when recognized by neighboring phagocytes [3]. PS ligation induces the release of transforming growth factor-β (TGF-β), an antiinflammatory cytokine that mediates the suppression of macrophage-mediated inflammation [4]. In *Hydra vulgaris*, an organism that stands at the base of metazoan evolution, the selective advantage provided by apoptosis lies in the fact that *Hydra* can survive recycling apoptotic cells by phagocytosis [5]. In unicellular organisms, it has been proposed that altruistic death benefits clonal populations of yeasts [6] and trypanosomatids [7–9]. Now we show that advantageous features of the apoptotic process can operate without death as the necessary outcome. *Leishmania* spp are able to evade the killing activity of phagocytes and establish themselves as obligate intracellular parasites. Amastigotes, responsible for disease propagation, similar to apoptotic cells, inhibit macrophage activity by exposing PS. Exposed PS participates in amastigote internalization. Recognition of this moiety by macrophages induces TGF-β secretion and IL-10 synthesis, inhibits NO production, and increases susceptibility to intracellular leishmanial growth.

Addresses: *Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo 05508-900, Brasil. †Divisão de Medicina Experimental, Instituto Nacional de Câncer, Rio de Janeiro 21230-120, Brasil. ‡Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21949-900, Brasil. §Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro 21045-900, Brasil.

¶These authors contributed equally to this work.

Correspondence: Marcello A. Barcinski
E-mail: barcinsk@inca.org.br

Received: **8 August 2001**
Revised: **17 September 2001**
Accepted: **10 October 2001**

Published: **27 November 2001**

Current Biology 2001, 11:1870–1873

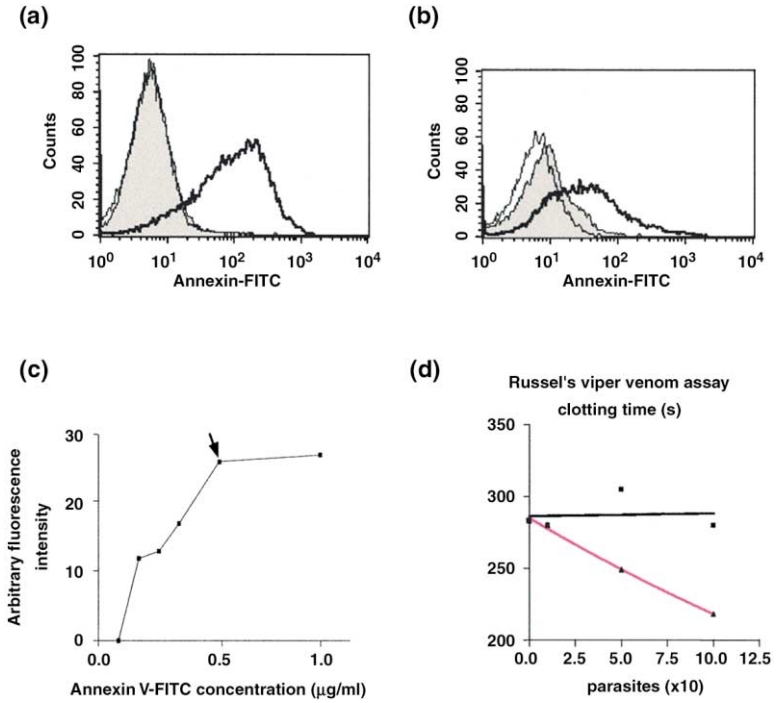
0960-9822/01/$ – see front matter

Results and discussion

PS exposure in amastigotes purified from murine footpad lesions was assessed by flow cytometry using FITC-labeled Annexin-V as a ligand. As in mammalian apoptotic cells, the binding of Annexin-V to the parasite was calcium dependent (Figure 1a). Inhibition of the binding of Annexin-V-FITC by an excess of unlabeled ligand (Figure 1b), by PS-exposing red blood cell vesicles and by phosphatidylcholine-phosphatidylserine, but not by phosphatidylcholine liposomes (data not shown) and the saturation of the binding curve (Figure 1c), confirmed the binding specificity. To confirm the presence of functional PS moieties on the amastigote surface, we measured the capacity of the parasite to induce procoagulant activity in the Russel's viper venom (RVV) assay [10]. Amastigotes yielded a dose-response curve (Figure 1d), while log-phase culture promastigotes, Annexin-V-negative forms of the parasite, were devoid of the activity. Promastigotes, the parasite form that multiplies inside the insect vector, are noninfective in the log-phase of cell growth [11] when cultured in vitro. Since amastigotes were purified from murine lesions, we considered the possibility that they contained adhered macrophage membranes. To minimize this possibility, amastigote purification included a step of continuous agitation that eliminates contaminating membranes [12]. Indeed, contamination was discarded by verification by flow cytometry that the amastigote preparation was free of macrophage membrane markers (H-2 class II antigens and CD11c) (data not shown).

To assess the role of PS in the amastigote/macrophage interaction, we analyzed the effects of masking this moiety on the amastigote surface with Annexin-V. Just as previously shown with apoptotic mammalian cells [13], in vitro infection of BALB/c peritoneal macrophages with amastigotes was significantly inhibited by Annexin-V (Figure 2a). Amastigote pretreatment with Annexin-V also yielded in vivo lesions that did not contain as many infected macrophages (Figure 2b, right panel). It is important to stress that Annexin-V does not affect parasite viability as analyzed by the MTT assay. This assay measures the cleavage by living, metabolically active cells of a tetra-

Figure 1



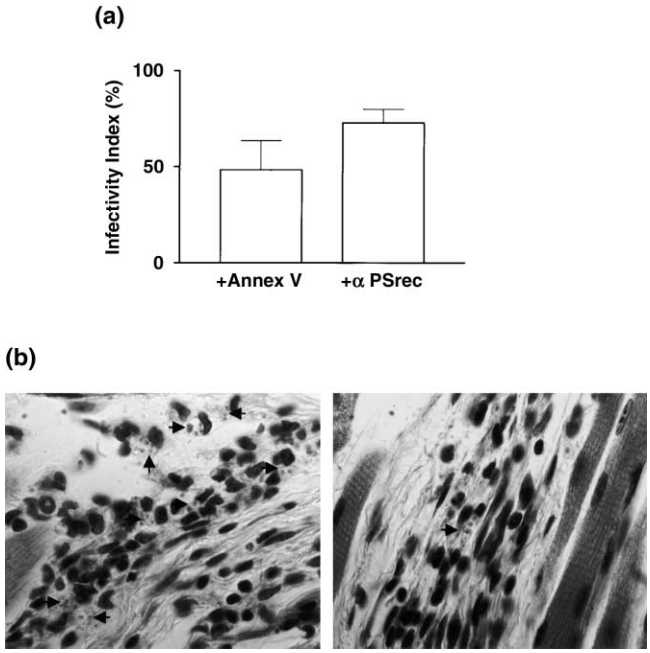
PS exposure in amastigotes. **(a–c)** Assessment by flow cytometry. (a) Lesion-purified amastigotes in Annexin-V binding buffer (100 mM HEPES, 150 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, and 1 mM $MgCl_2$ [pH 7.4]) were incubated at room temperature for 15 min with 10 μg/ml PI alone (thin line), PI plus 0.5 μg/ml Annexin-V-FITC (Clonentech) (bold line), and PI plus Annexin-V plus 5 mM EGTA (fill gray). (b) Same as in (a), but in the presence of 0.50 μg/ml unlabeled Annexin-V (fill gray). (c) Saturation of the Annexin-V binding curve. Cells were incubated at room temperature for 15 min with 10 μg/ml PI plus 0.125 μg/ml–1.000 μg/ml Annexin-V-FITC. The arrow indicates the working dilution used in all other labeling experiments. Data were collected in a BD FACScan and were analyzed by Cellquest. In experiments using EGTA, the Annexin-V binding buffer was made with 3 mM $CaCl_2$. A total of 10,000 events were harvested from each sample. **(d)** Assessment by procoagulant activity. The effect of purified amastigotes (triangle) or promastigotes (square) on the RVV-induced in vitro blood coagulation assay. The RVV assay was run by incubating 100-μl aliquots of citrated platelet-poor plasma (pooled from three normal volunteers) at 37°C in a water bath. After 2 min, 30 μl (12 ng) RVV (Sigma) was added, followed by a 100-μl aliquot of the desired number of cells and 100 μl of a 25 mM concentration of $CaCl_2$. The time required to form a firm fibrin clot was then determined. For the positive control, a standardized amount of cephalin (STA standard-Diagnostica Stago) was used.

zolium salt and has been used for assessing the viability of *Leishmania* spp parasites [14]. The participation of PS in amastigote internalization was further confirmed by showing inhibition by a monoclonal antibody (mAb 217G8E9) that identifies a recently described receptor, expressed on the surface of macrophages, fibroblasts, and epithelial cells, which recognizes apoptotic cells and clearly distinguishes PS from other lipids [15] (Figure 2a). Albeit significant and reproducible, the rather low inhibitory effect of this IgM antibody probably reflects its low affinity for the PS receptor [3]. This last result implies that the same macrophage receptor can recognize PS on the surface of amastigotes and apoptotic mammalian cells.

Next, to demonstrate that PS recognition deactivates macrophages, making them more amenable for parasite growth, we fed murine peritoneal macrophages with irradiation-induced apoptotic BALB/c thymocytes (30%–60% Annexin-V positive) prior to infection with amastigotes and compared their infectivity index (percent of infected macrophages multiplied by the average number of amastigotes in infected macrophages) with that obtained following infection of untreated macrophages. As seen in Figure 3a, the infectivity index was much higher in macrophages previously fed with apoptotic cells than in untreated macrophages. When PS is present on the parasite surface, the same macrophage-inhibitory activity is observed. Pretreatment of amastigotes with Annexin-V not only inhibited internalization, as shown above, but also reduced the parasite's intracellular growth rate, indicating a higher growth-limiting activity of the phagocyte infected with amastigotes with less PS available on their surface, as compared to an infection by normal PS-exposing parasites (data not shown). Remarkably, local inflammatory infiltrates were significantly larger in mice infected with amastigotes with PS moieties masked by pretreatment with Annexin-V when compared to those infected with nontreated amastigotes. The observed effects of Annexin-V confirm that PS molecules are exposed on the parasite surface and participate in amastigote internalization and on the inhibition of macrophage proinflammatory activity.

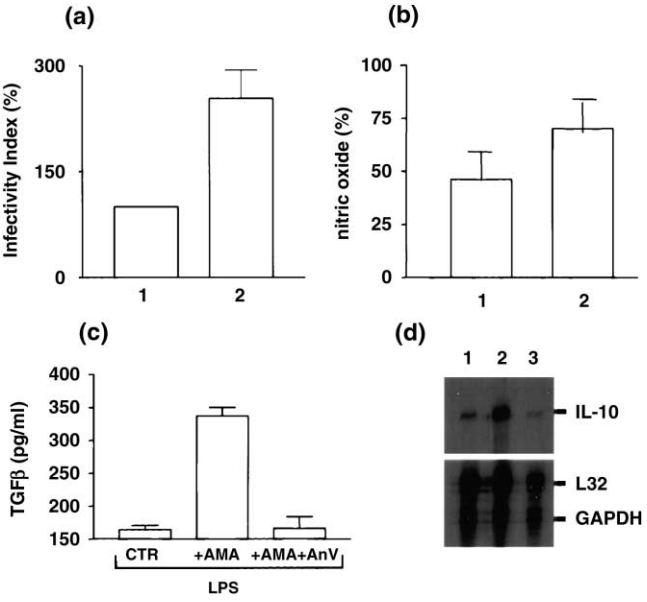
Since liposomes containing PS selectively inhibit leishmanicidal activity [16], we assessed macrophage production of NO, the most effective microbicidal agent, following internalization of amastigotes and apoptotic mammalian cells. Amastigote internalization is not able, by itself, to induce NO synthesis by peritoneal macrophages [17]. However, with γIFN plus LPS as the inducing agent, amastigotes and irradiated thymocytes exerted a strong inhibitory effect on NO production by stimulated macrophages (Figure 3b). TGF-β, which has been shown to

Figure 2



Inhibition of macrophage infection. **(a)** In vitro inhibition by Annexin-V and anti-PS receptor. The infectivity index of resident macrophages was assessed after 2 hr of infection with purified amastigotes in the presence of 0.50 μg/ml Annexin-V (+Annex V) or 200 μg/ml monoclonal anti-PS receptor (+αPsrec) IgM antibody (mAb 217G8E9). The bars represent mean ± SD of three experiments. p values were < 0.005. **(b)** The effect of Annexin-V on in vivo infection. BALB/c mice footpad sections 48 hr after infection with amastigotes preincubated (right panel, 11.2 ± 1.7% infected macrophages), or not (left panel, 41.5 ± 6.8% infected macrophages), with Annexin-V. The hind footpad of BALB/c mice was inoculated with 3×10^6 amastigotes. A total of 48 hr after inoculation, the whole hind foot was cut off and was immediately fixed in buffered formalin for 12 hr. The foot was then cut longitudinally into two pieces, which were decalcified in 10% EDTA solution for 1 week. The fragments were then embedded in paraffin, and a 4-μm section was stained with heaematoxylin and eosin. Each slide was examined using a Zeiss Axiolab microscope and 1000× magnification. Head arrows indicate amastigote nests inside macrophages. The panels represent one out of three animals.

Figure 3



Modulation of macrophage activity by PS-exposing cells. **(a)** Exacerbation of in vitro infection by apoptotic thymocytes. A total of 2×10^6 pooled resident BALB/c macrophages/ml were plated in Lab-Tek chambers at 37°C. After 16 hr, cells were incubated with 2×10^6 amastigotes/ml at 34°C, with or without a previous 2-hr incubation with apoptotic thymocytes (6- to 8-hr culture after irradiation with 600 rads), and the infectivity index (percentage of infected macrophages multiplied by the average number of amastigotes in infected macrophages) was microscopically assessed after 72 hr of culture. The bars represent mean ± SD of three experiments. p values were <0.005. **(b)** Inactivation of macrophage NO production induced by amastigotes and apoptotic thymocytes. A total of 2×10^6 peritoneal resident macrophages were activated with 100 ng/ml LPS plus 0.10 ng/ml IFN-γ in the absence or presence of 2×10^6 amastigotes ($p = 0.002$) and in the presence of BALB/c apoptotic thymocytes ($p = 0.021$). After 24 hr of culture, supernatants were collected, and the production of NO was assessed by the Griess reaction. The figure represents mean ± SD of three experiments. **(c)** Induction of macrophage TGF-β production by amastigotes. Thyoglicolate-induced BALB/c macrophages were activated with 50 ng/ml LPS. Supernatants from uninfected (CTR), and infected (in the absence, +AMA, or in the presence, +AMA+AnV, of 0.50 μg/ml Annexin-V) macrophages were assayed for active TGF-β by ELISA (DuoSet kit from R&D) after 72 hr of culture in serum-free CCM1 medium. The concentration of TGF-β in each experiment was determined by comparison with a curve generated from TGF-β standards. The panel represents mean ± SD of three independent experiments. **(d)** IL-10-specific message in macrophages in the presence of amastigotes and apoptotic thymocytes assessed by an RNase protection assay. RNAs were extracted from noninfected resident peritoneal macrophages (lane 1) and after 2 hr of incubation with amastigotes (lane 2) or irradiated thymocytes (lane 3). The IL-10 message was detected with the use of an RNase protection assay kit (Pharmingen). Fragments of housekeeping genes (L32 and GAPDH) are shown as internal controls. The panel represents one out of three experiments.

induce leishmanial growth [18], has also been described as the main inhibitor of macrophage inflammatory activity upon recognition of apoptotic cells [4]. Now we show that amastigotes are indeed capable of activating TGF-β and that this activity is abrogated by pretreatment with Annexin-V (Figure 3c). This result indicates that, also in the present situation, TGF-β secretion is dependent on the macrophages' recognition of PS moieties at the parasite surface [15]. Despite their similarities in the ability to inactivate macrophages, making them more susceptible to parasite growth, amastigotes, as opposed to thymocytes, induced IL-10 synthesis (Figure 3d). This result reflects differential macrophage signaling by apoptotic cells that will be destroyed and by microorganisms that will survive and multiply inside the phagocyte.

Taking advantage of the ability of apoptotic cells to make host macrophages more susceptible to parasite intracellular parasite multiplication seems to be an adaptive strategy

of pathogenic trypanosomatids. While *Trypanosoma cruzi* uses apoptotic host T lymphocytes for macrophage inactivation and consequent persistence in the mammalian host [19], *Leishmania (L) amazonensis*, as an obligate intramacrophagic parasite and thus in need of a mechanism of immediate macrophage inactivation, uses an apoptotic-like feature of its own cells for a similar purpose.

Acknowledgements

We thank Dr. Valerie Fadok for the monoclonal anti-PS receptor antibody (mAb 217G8E9). This work was supported by grants from the Fundação de Amparo à Pesquisa de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Tecnológico e Científico (CNPq), Fundação Oswaldo Cruz, and Instituto Nacional do Câncer.

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